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Disruption of Thiamine Uptake and Growth of Cells by Feline Leukemia Virus Subgroup A

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Feline leukemia virus (FeLV) is still a major cause of morbidity and mortality in domestic cats and some wild cats despite the availability of relatively effective vaccines against the virus. FeLV subgroup A (FeLV-A) is transmitted in natural infections, and FeLV subgroups B, C, and T can evolve directly from FeLV-A by mutation and/or recombination with endogenous retroviruses in domestic cats, resulting in a variety of pathogenic outcomes. The cell surface entry receptor for FeLV-A is a putative thiamine transporter (THTR1). Here, we have addressed whether FeLV-A infection might disrupt thiamine uptake into cells and, because thiamine is an essential nutrient, whether this disruption might have pathological consequences. First, we cloned the cat ortholog of the other of the two known thiamine transporters in mammals, THTR2, and we show that feline THTR1 (feTHTR1) and feTHTR2 both mediate thiamine uptake, but feTHTR2 does not function as a receptor for FeLV-A. We found that feTHTR1 is widely expressed in cat tissues and in cell lines, while expression of feTHTR2 is restricted. Thiamine uptake mediated by feTHTR1 was indeed blocked by FeLV-A infection, and in feline fibroblasts that naturally express feTHTR1 and not feTHTR2, this blockade resulted in a growth arrest at physiological concentrations of extracellular thiamine. The growth arrest was reversed at high extracellular concentrations of thiamine. Our results show that FeLV-A infection can indeed disrupt thiamine uptake with pathological consequences. A prediction of these experiments is that raising the plasma levels of thiamine in FeLV-infected cats may ameliorate the pathogenic effects of infection.

Feline leukemia virus (FeLV) is still a major cause of morbidity and mortality in domestic cats and is also pathogenic in some wild cat species (1–3). Although FeLV vaccines are now available, they do not provide protection in all cats, and the duration of protection is unclear (1). FeLV subgroup A (FeLV-A) viruses are found in all naturally infected cats, and it is this subgroup of FeLV that is highly transmissible in nature (4). Studies in infected cats and in cultured cells have demonstrated that other FeLV subgroups (which include B, C, and T) can evolve directly from FeLV-A (5–7). Thus, chronically infected cats often harbor a mix of both FeLV-A and other FeLV subgroups. Subgroups B, C, and T have all been linked to pathogenic outcomes, including lymphoma and leukemia, aplastic anemia, and immunodeficiency, respectively. Because FeLV-A is the progenitor for these pathogenic forms, it has been difficult to precisely define the contribution of FeLV-A to clinical outcome in FeLV-infected cats. However, immunosuppression appears to be one early effect of FeLV-A infection.

Retroviral envelope proteins can cause downregulation of their cognate cellular receptors by a variety of mechanisms, and this has serious pathogenic consequences in cases where there is no compensatory mechanism to offset the block in transporter function. In cats viremic with feline leukemia virus subgroup C (FeLV-C), the envelope protein acts as a dominant negative inhibitor preventing display and/or function of the cellular receptor FLVCR1, a heme export protein (8). The disruption of FLVCR1 function causes a toxic accumulation of free heme in developing erythroid progenitor cells, resulting in a loss of erythroid cell progeny (8). This type of erythroid depletion is a form of pure red cell aplasia and is fatal in cats infected with FeLV-C. While FeLV-C is known to infect other cell types that express the FLVCR1 receptor, the infection of early cells of the erythroid lineage is most detrimental because these cells lack globin and other heme-binding proteins and do not have a secondary transport system to compensate for the disruption in FLVCR1 function. These findings highlight the variable effects that FeLV infection may have on distinct cell populations and on their ability to tolerate retroviral envelope-mediated disruption of their cellular receptors.

Here, we have investigated the possibility that FeLV-A interaction with its cellular receptor might have a role in FeLV-A persistence and pathogenesis. The FeLV-A receptor has been identified (9) and shows high sequence similarity to a human thiamine transport protein (huTHTR1; SLC19A2) (10–12). On this basis, the feline FeLV-A receptor was named feTHTR1, although its transport function had yet to be defined. The huTHTR1 protein is one of two known thiamine transporters found in humans; the other is the product of the SLC19A3 gene (huTHTR2) (13, 14). These two proteins are the only thiamine transporters identified in mammals to date. Thiamine (vitamin B_1) is an essential cofactor for several enzymes, and humans and other mammals cannot synthesize thiamine *de novo* and are therefore completely dependent on obtaining thiamine from exogenous sources through intestinal absorption. Therefore, functional THTR1 and THTR2 are vital to maintaining adequate cellular levels of thiamine, and disruption of thiamine uptake by FeLV-A infection might have pathogenic consequences.

Mutations in the gene encoding huTHTR1 have been directly linked to thiamine-responsive megaloblastic anemia syndrome (TRMA) in humans (10, 12). TRMA is an autosomal recessive disorder characterized by megaloblastic anemia, pancytopenia,

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diabetes mellitus, and sensory neuronal deafness (15). *In vitro* experiments with epithelial cells from TRMA patients have shown that these cells have decreased thiamine-dependent enzymatic activity and undergo apoptosis when passaged at physiological levels of thiamine, which do not affect growth and survival of normal epithelial cells (16).

Mutations in the gene encoding huTHTR2 have been linked to thiamine metabolism dysfunction syndrome 2 (THMD2) in humans (17, 18). THMD2, also known as biotin-responsive basal ganglia disease, is an autosomal recessive metabolic disorder characterized by episodic encephalopathy, generalized dystonia, and epilepsy. Interestingly, THTR2 knockout mice have no disease phenotype, unlike murine THTR1 knockout mice, which exhibit a TRMA-like phenotype. The results of these studies show that THTR1 and THTR2 serve specific roles in thiamine uptake and homeostasis that are not completely overlapping.

To better understand the role of FeLV-A receptor interactions in FeLV pathogenesis, we cloned the feline THTR2 cDNA and examined the transport and virus receptor properties of feTHTR1 and feTHTR2. Both proteins specifically transport thiamine into cells, but only feTHTR1 serves as a receptor for FeLV-A. We find that several feline cell lines and tissues express feTHTR1, but not feTHTR2, and FeLV-A infection of such cell lines can arrest their growth at physiologically relevant thiamine concentrations. Our results suggest that inhibition of thiamine uptake by FeLV-A in some cat tissues may contribute to FeLV pathogenicity.

(Some of these data were included in a dissertation submitted by Ramon Mendoza to the University of Washington in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Materials. [^{3}H]thiamine (specific activity, 1.4 Ci/mmol; radiochemical purity, >98%) was purchased from Moravek Biochemicals (Brea, CA). Custom-made thiamine-deficient Dulbecco's modified Eagle medium (DMEM) was purchased from HyClone (Logan, UT). TRIzol reagent and DNA oligonucleotide primers were obtained from Invitrogen (Carlsbad, CA). Dialyzed fetal bovine serum was obtained from Gibco (Grand Island, NY).

RNA extraction from feline tissues and cultured cells. Ten- to 50-mg tissue samples from uninfected domestic cats, which had been frozen at −80°C, were each added to 1 ml of TRIzol (Life Technologies, Grand Island, NY) and homogenized for 1 min using a handheld tissue homogenizer (Polytron model PT-1200; Kinematica, Lucerne, Switzerland), and RNA was prepared by using the manufacturer's protocol. For cell lines, the cells were harvested and RNA was prepared using TRIzol. RNA was quantified by measuring absorbance at 260 nm.

Identification and cloning of the feTHTR2 cDNA. Feline cDNA was made from liver-derived RNA by reverse transcription with SuperScript III (Life Technologies) and oligo(dT) as the primer. Primers (5′-CAGAG ATAACAAATGAGATC-3′ and 5′-AGTCATGATGGTCTGAATCACCA AGGC-3′) were designed that match conserved regions in mouse and human THTR2 cDNAs and used to amplify a 1-kb fragment corresponding to a sequence within the coding region of feTHTR2. To obtain a full-length cDNA, rapid amplification of cDNA ends (RACE) using primers designed against sequences in the 1-kb fragment was employed. The complete sequence of the feTHTR2 cDNA was determined by capillary electrophoresis using an Applied Biosystems 3730xl DNA analyzer (Life Technologies). For expression studies, the feline cDNA was subcloned into the retroviral vector LXSN (19). For this purpose, the feTHTR2 cDNA was digested with BamHI and a 2.8-kb fragment containing the feTHTR2 coding region was inserted into the BamHI site of LXSN to generate the L(feTHTR2)SN vector.

Reverse transcriptase PCR (RT-PCR) for detection of feTHT1 and feTHTR2. First-strand cDNA synthesis was carried out using SuperScript III (Life Sciences) and oligo(dT) as the primer. RNase H was then added to digest the remaining RNA. The feTHTR1 cDNA was amplified using the primer pairs feTHTR1-13 (5′-GACTGCTGGCCATTCAGTTCTTG-3′) and feTHTR1-14 (5′-GGTGGCACTCCGACAGTAACTCG-3′). The feTHTR2 cDNA was amplified using the primer pairs feTHTR2-9 (5′-G TGCAGGCTCTCTGTTTCTCATG-3′) and feTHTR2-10 (5′-GCGCTC CACACTCAGATTAACTG-3′). The primer pairs for the amplification of feline GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 57f and 138R, have been described (20). Primer pairs were tested for specificity on dilutions of plasmids from 10 to 10^6 copies, as determined by spectrophotometer measurements at 260 nm. Single-round PCR consisted of 35 cycles of 30 s of template denaturation at 94°C, 30 s of primer binding at 58°C, and 30 s of elongation at 72°C, with an initial 5-min denaturation at 94°C and a final 5-min elongation at 72°C for completion. Amplification products were analyzed by gel electrophoresis using 2% agarose.

Cell culture. AH927 feline fibroblasts (originally from W. Nelson-Rees), *Mus dunni* tail fibroblasts (MDTF) (21), and 293T (293/*tsA1609*neo) simian virus 40 (SV40) T-antigen-transformed human embryonic kidney cells (22) were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U penicillin per ml, 100 mg streptomycin per ml, and 0.25 mg amphotericin B per ml.

MDTF cell lines expressing receptor proteins, MDTF-feTHTR1, MDTF-huTHTR1, and MDTF-fePit1, have been described elsewhere (23). MDTF-feTHTR2 cells were generated for this study in a similar manner as the other MDTF cell derivatives. Briefly, all cDNAs were expressed using the retroviral expression vector LXSN. Virus was made from the plasmid forms of the vectors and was used to transduce the MDTF cells. All cell populations were selected and maintained in 0.6 mg G418 per ml to select for expression of the *neo* gene carried by the LXSN vector and to ensure that all cell populations expressed the relevant transgenes.

AH927 feline cell lines LEAH and LBAH, which express Env proteins from FeLV-A strain 61E and FeLV-B strain EEZZ, respectively, have been described (24).

Virus production and infection assays. Pseudotyped virus was made by calcium phosphate transfection of 293T cells with a construct expressing FeLV-61E-gag-pol (61E-LTR-ΔΨ-gag-pol), a murine leukemia virus (MLV)-derived reporter vector (pRT43.2Tnlsβgal-1) that expresses β-galactosidase (β-Gal), and the desired FeLV envelope construct, as described previously (25). Replication-competent FeLV-A strain 61E (26) and FeLV-B strain EEZZ (6) were generated by transfection of plasmids containing the respective proviruses into 293T cells. Virus was harvested after 48 h, filtered using 0.2-μm-pore-size filters, and frozen at −70°C.

Vector infection assay. Virus susceptibility was assayed as described previously (23). Briefly, target cells were seeded at 2×10^4 cells/well in 24-well plates the day before infection. The next day, the culture medium was replaced with medium containing 4 μg Polybrene per ml and the β-Gal-expressing viral vector was added. At 48 h postinfection, cells were stained for β-Gal expression.

Thiamine uptake studies. Thiamine uptake was measured in confluent monolayers of cells seeded 2 days earlier in 24-well plates at 3×10^4 cells per well. Uptake was measured in Krebs-Ringer (KR) buffer (133 mM NaCl, 4.93 mM KCl, 1.23 mM $MgSO_4$, 0.85 mM $CaCl_2$, 5 mM glucose, 5 mM glutamine, 10 mM HEPES, and 10 mM MES [morpholineethanesulfonic acid], pH 7.4). Incubations were performed at 37°C for 7 min. Tritium-labeled thiamine ([^{3}H]thiamine), with or without inhibitors, such as unlabeled thiamine, or other compounds of interest, was added to the incubation medium at the onset of the uptake experiment, and the reaction was terminated by the addition of 2 ml of ice-cold KR buffer followed by immediate aspiration. Cells were then rinsed twice with ice-cold KR buffer and were lysed using 0.5 ml of lysis buffer (0.1% NP-40, 500 mM NaCl, 5 mM EDTA) per well. Radioactivity of the lysate was measured using scintillation fluid, and protein content was measured using a Pierce bicinchoninic acid (BCA) protein quantitation kit (Pierce, Rockford, IL).

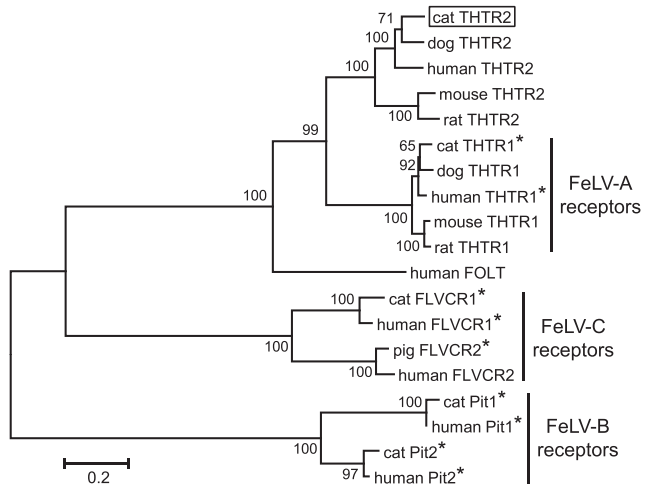

FIG 1 Phylogenetic relationship of FeLV receptors and related proteins, including the proposed cat THTR2 protein (boxed). Asterisks indicate the receptor orthologs that have been shown to be functional receptors for the FeLV subgroup viruses (9, 23, 27–31). The protein sequence of the human folate transporter (FOLT; SLC19A1) isoform 1 is included. All protein sequences, with the exception of cat THTR2, were RefSeq versions obtained from GenBank in September 2012. A phylogenetic tree was generated using the neighbor-joining algorithm of MEGA 5.05 (32) with default settings. Bootstrap test values for 500 replications are shown at the tree branches, and the scale bar indicates evolutionary distance in amino acid substitutions per site.

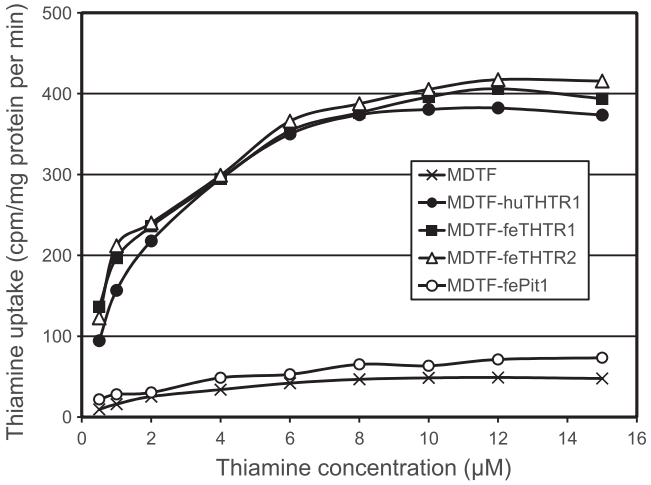

FIG 2 Feline THTR1 and THTR2 are thiamine transporters. MDTF cells expressing the indicated proteins were incubated with [^{3}H]thiamine at the indicated concentrations for 7 min, and cell-associated radioactivity was measured. Results are the means of two independent experiments and are expressed in cpm per mg cell protein per min.

Nucleotide sequence accession number. The complete sequence of the feTHTR2 cDNA has been deposited into GenBank under the accession number JX880025.

RESULTS

Cloning of the feline THTR2 cDNA. In mice and humans, there are only two known thiamine transport proteins, THTR1 and THTR2. To determine whether cats have a THTR2 ortholog, in addition to the known THTR1 ortholog, we attempted to amplify and clone feTHTR2 cDNA. Using consensus DNA primers based on the known THTR2 coding sequences, we were able to isolate a 1-kb sequence with high similarity to other THTR2 sequences but with less similarity to THTR1 coding sequences. We then used RACE to clone the 5′ and 3′ ends of the cDNA and generate a complete cDNA clone (GenBank accession number JX880025). This clone is 2,700 bp long and contains an open reading frame that encodes a protein of 495 amino acids in length. A phylogenetic analysis of this protein sequence and other known THTR protein and related sequences (Fig. 1) shows that the cDNA sequence we cloned encodes a protein that clusters with other THTR2 protein sequences, indicating that the cDNA sequence we cloned is indeed that of feline THTR2.

Feline THTR2 does not function as a receptor for FeLV-A. To test whether the feTHTR2 protein could function as a receptor for FeLV-A, the feTHTR2 cDNA was cloned into the LXSN retroviral expression vector (19), and virus was generated from this plasmid and used to transduce MDTF cells to make MDTF-feTHTR2 cells. The MDTF-feTHTR2 cells were not permissive to infection by an FeLV-A pseudotype retroviral vector (vector titer, <1 FFU/ml; $n = 4$), whereas MDTF-feTHTR1 cells tested in parallel were highly permissive, showing vector titers of 2×10^5 FFU/ml (mean of four experiments). For comparison, the titer of the vector on feline fibroblasts tested in parallel was 5×10^5 FFU/ml (mean of four experiments), confirming that feTHTR1 functions as an effective receptor for FeLV-A. These experiments, together with those described below showing that the engineered MDTF-feTHTR2 cells do express feTHTR2, show that feTHTR2 does not function as a cell entry receptor for FeLV-A.

THTR proteins are predicted to span the cell surface membrane 12 times and to have cytoplasmic amino and carboxy termini, based on their hydropathy profiles and on experimental topological data for the related human folate transporter protein (FOLT; SLC19A1) (33). We compared the amino acid sequences of the predicted external loops of feTHTR1 and huTHTR1, which both serve as receptors for FeLV-A, to those of feTHTR2, which does not, to determine if alterations in specific extracellular loops might account for the difference in receptor function. While the extracellular loops of feTHTR1 and huTHTR1 are quite similar, with only loops 4 and 5 showing amino acid differences of 3 residues and 1 residue, respectively, all of the extracellular loops of feTHTR2 differ from those of feTHTR1 and huTHTR1 by 3 to 11 residues, such that any combination of these extracellular loops might be responsible for the difference in THTR protein function as a receptor for FeLV-A.

Both feTHTR1 and feTHTR2 are thiamine transporters and have activities similar to that of the huTHTR1 protein. To determine whether the feline THTR proteins function as thiamine transporters, we measured uptake of [^{3}H]thiamine by MDTF cells stably expressing feTHTR1, feTHTR2, huTHTR1, or the feline inorganic phosphate transporter Pit1 (fePit1), which would not be expected to transport thiamine. All cDNAs were expressed using the retroviral expression vector LXSN in an attempt to ensure comparable levels of protein expression. Thiamine transport was detected in unmodified MDTF cells, most likely reflecting the expression of endogenous mouse THTR proteins (Fig. 2). MDTF cells expressing feTHTR1, feTHTR2, or huTHTR1 exhibited similar thiamine uptake activities, which were well above (~6-fold) the uptake activity of unmodified MDTF cells (Fig. 2). Thiamine uptake by MDTF cells expressing fePit1 was similar to that of unmodified MDTF cells, as expected. Thiamine uptake was satu-

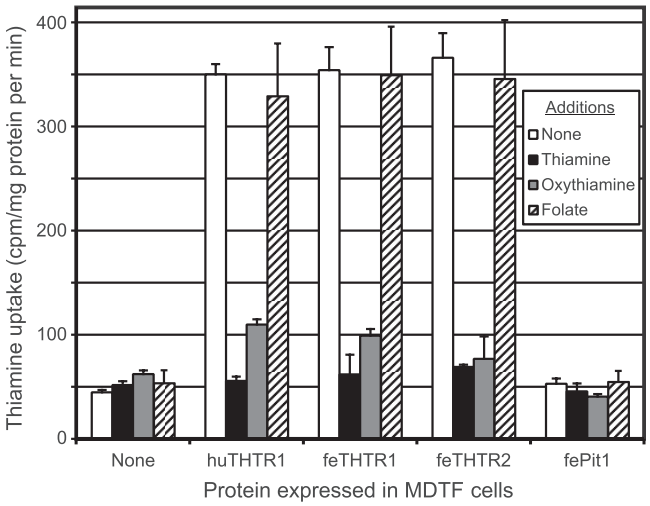

FIG 3 Thiamine uptake by cells expressing feTHTR1, feTHTR2, and huTHTR1 is blocked by excess unlabeled thiamine or oxythiamine, but not by folate. Cells were incubated with 4 μM [^{3}H]thiamine plus the indicated additional compounds for 7 min, and cell-associated radioactivity was measured. Results are the means ± standard deviations of two independent experiments and are expressed in cpm per mg cell protein per min.

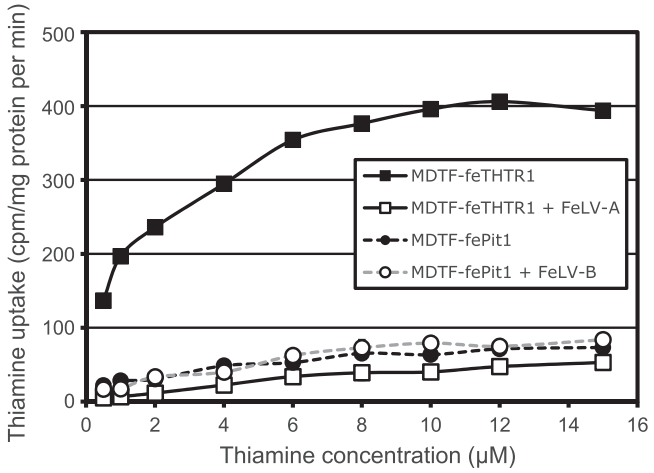

FIG 4 Thiamine uptake by feTHTR1 is blocked in cells infected with FeLV-A. Cells were incubated with 4 μM [^{3}H]thiamine for 7 min, and cell-associated radioactivity was measured. Results are the means of three independent experiments and are expressed in cpm per mg cell protein per min.

rable at high concentrations of thiamine, and the K_m for thiamine uptake by the THTR proteins was similar, ~1 to 2 μM. These experiments indicate that all three THTR proteins exhibit similar thiamine transport activities, although we have not proven that the proteins are made at the same levels.

Transport by feTHTR1 and feTHTR2 is specific for thiamine and closely related molecules. To test the relative specificity of feTHTR proteins for thiamine, we assayed thiamine uptake in the presence of oxythiamine, a known thiamine transport inhibitor, or folate, which is transported by the related folate transport protein, SLC19A1. Cells stably expressing the receptors of interest were incubated with 4 μM radiolabeled [^{3}H]thiamine in the presence of 1 mM unlabeled thiamine, oxythiamine, or folate, and [^{3}H]thiamine uptake was measured (Fig. 3). The addition of unlabeled thiamine or oxythiamine dramatically inhibited [^{3}H]thiamine uptake to levels similar to those of MDTF and MDTF-fePit1 cells, while the addition of folate had no effect. These data indicate that feTHTR1 and feTHTR2 specifically transport thiamine and other closely related molecules, but not folate.

Thiamine uptake in MDTF-feTHTR1 cells is inhibited by FeLV-A infection. To explore the effect of FeLV-A infection on thiamine uptake mediated by feTHTR1, MDTF-feTHTR1 cells were infected with the replication-competent FeLV-A (strain 61E) retrovirus (26). As a control, MDTF-fePit1 cells were infected with a replication-competent virus of another subgroup that uses the fePit1 receptor, FeLV-B (strain EEZZ) (6). Virus production, measured by FeLV gag polyprotein (p27), was detected in culture medium from MDTF-feTHTR1 cells infected with FeLV-A at 7 days after infection, indicating productive FeLV-A replication. To determine if these cells had established superinfection interference, the cells were infected with a β-Gal-expressing viral vector pseudotyped with the FeLV-A envelope protein or with an amphotropic murine leukemia virus (A-MLV) envelope protein. The MDTF-feTHTR1 cells infected with FeLV-A were completely resistant to reinfection by the FeLV-A pseudotyped vector, even when exposed to 10^5 infectious virus particles, but remained permissive to infection by the A-MLV pseudotyped virus, which uses the unrelated Pit2 protein as a receptor (data not shown). In parallel to these results, MDTF-fePit1 cells infected with FeLV-B were completely resistant to reinfection by FeLV-B pseudotyped virus but remained permissive to A-MLV pseudotyped vector infection (data not shown). These results show that superinfection interference was established in these cells, which thus could be used to assess the impact of infection on transport function.

In thiamine uptake experiments (Fig. 4), MDTF-feTHTR1 cells exhibited a high rate of thiamine uptake, which was dramatically reduced in MDTF-feTHTR1 cells infected with FeLV-A. The rate of thiamine uptake by FeLV-A-infected cells was similar to that of MDTF cells that did not express the feTHTR1 receptor, including both MDTF-fePit1 cells and MDTF-fePit1 cells infected with FeLV-B. As expected, chronic infection with FeLV-B had little effect on thiamine transport by MDTF-fePit1 cells. These results show that FeLV-A infection of cells in which thiamine uptake is mediated primarily by feTHTR1 can severely disrupt thiamine uptake.

Tissue-specific expression pattern of feTHTR1 and feTHTR2. Expression profiles of THTR1 and THTR2 in human and mouse tissue indicate that the THTR1 gene is ubiquitously expressed, while THTR2 has a much narrower expression profile (12, 13, 34, 35). Cells primarily expressing feTHTR1 might be particularly susceptible to toxic effects of FeLV-A disruption of thiamine uptake. To determine if feTHTR1 and feTHTR2 exhibit similar expression patterns in cats, we developed a sensitive RT-PCR assay using gene-specific primers to detect mRNA transcripts of feTHTR1 or feTHTR2. When tested on serial dilutions of plasmid template, the gene-specific primers amplified their targeted templates down to 100 copies and did not amplify their counterpart template even at copy numbers as high as 10^6 (data not shown).

We examined mRNA expression with this RT-PCR method using total RNA extracted from various feline cell lines and tissues, including MDTF-feTHTR1 and MDTF-feTHTR2 cell lines (Fig. 5). The proper RT-PCR products were detected in mRNA from the feTHTR1- and feTHTR2-expressing MDTF cells but were not

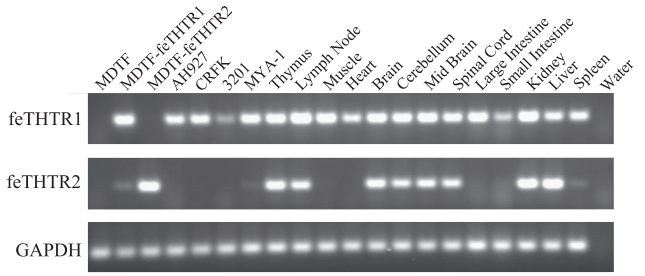

FIG 5 Detection of feTHTR1 and feTHTR2 mRNA in feline cell lines and tissues. RT-PCR products from cell lines and tissue for feTHTR2 and feTHTR2 mRNA levels were analyzed on an agarose gel as described in Materials and Methods. Data shown are representative of several independent experiments. GAPDH is shown as a control for relative input across samples.

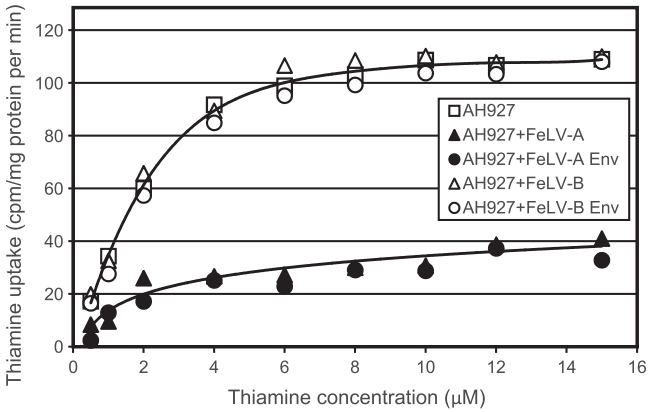

FIG 6 Thiamine uptake by AH927 feline embryo fibroblasts is inhibited by FeLV-A Env expression. Cells were incubated with 4 μM [^{3}H]thiamine for 7 min, and cell-associated radioactivity was measured. Results are the means of three independent experiments and are expressed in cpm per mg cell protein per min.

detected in mRNA from the parental MDTF cells. The feTHTR1 transcript was detected in AH927 feline embryo fibroblasts, MYA-1 (36) and 3201 (37) feline T-cell lines, and the CRFK feline kidney cell line (38); however, no feTHTR2 RNA was detected in any of these cell lines using RT-PCR. feTHTR1 transcripts were found in all feline tissues tested. In contrast, feTHTR2 mRNA was detected in only a subset of tissues, i.e., thymus, lymph node, brain, spinal cord, kidney, and liver, with a very low signal detected in spleen. feTHTR2 RNA was not detected in muscle, heart, or large or small intestine.

Infection by FeLV-A disrupts thiamine transport in feline fibroblasts and impairs their growth. To further investigate the effects of FeLV-A infection on feTHTR1-mediated thiamine transport by feline cells, we used AH927 feline fibroblasts that naturally express feTHTR1 but not feTHTR2 (Fig. 5). In addition to studying cells chronically infected with FeLV-A, or FeLV-B as a control, we used previously described AH927 cell lines that stably express the Env proteins from FeLV-A or FeLV-B (LEAH and LBAH, respectively) (24) to examine whether the FeLV-A Env protein alone could disrupt thiamine transport activity. Thiamine uptake was dramatically reduced in AH927 cells infected with FeLV-A or expressing FeLV-A Env compared to unmodified AH927 cells (Fig. 6). In contrast, thiamine transport was not affected in AH927 cells infected with FeLV-B or expressing FeLV-B Env compared to unmodified AH927 cells (Fig. 6). These results show specific blockade of thiamine uptake by the FeLV-A Env protein.

To determine whether disruption of thiamine transporter function has any physiological effect on AH927 cells, we measured cell growth over a range of thiamine concentrations that might be found in blood. Whole blood levels of thiamine measured in 29 outbred domestic cats ranged from 60 to 270 nmol/liter, with a median value of 150 (39). However, ~90% of this thiamine is sequestered in red blood cells as thiamine diphosphate, thus reducing the available thiamine concentration in plasma by at least 5-fold (assuming an hematocrit of ≤50%) to a median value of ≤30 nM. We therefore assayed for cell growth by plating AH927 cells infected with FeLV-A or FeLV-B at thiamine concentrations of 30 nM to 33 μM (Fig. 7). At 30 nM thiamine, AH927 cells grew rapidly from 2×10^3 to almost 10^6 per well. In contrast, AH927 cells infected with FeLV-A grew rapidly initially but plateaued at 2×10^4 per well 2 days after seeding and grew no further. Growth of AH927 cells infected with FeLV-B was similar to that of uninfected AH927 cells. At intermediate thiamine concentrations, FeLV-A-infected AH927 cells grew significantly less well than did uninfected AH927 cells, but the infected cells still continued to grow. At 33 μM thiamine, similar to the thiamine concentration of standard culture medium (e.g., 12 μM for DMEM), the growth rates and final cell density of AH927 cells and those infected with FeLV-A or FeLV-B were similar, suggesting that thiamine entry into cells by passive or active mechanisms other than high-affinity transport was sufficient to maintain cell growth at this high thiamine concentration despite disruption of feTHTR1 function by FeLV-A. These data indicate that growth in cats of cells that express feTHTR1 and not feTHTR2 would be negatively impacted by FeLV-A infection at physiologic concentrations of thiamine.

DISCUSSION

Only two related thiamine transporters are known in mammals, THTR1 and THTR2. Here, we have cloned the feline THTR2 cDNA and show that both the feline THTR1 and THTR2 proteins specifically transport thiamine with kinetics and transport rates similar to those of human THTR1. Infection by FeLV-A was associated with disruption of thiamine transport in both feline cells and nonfeline cells engineered to express feTHTR1. Furthermore, feline fibroblast cells infected with FeLV-A did not grow under conditions of limiting thiamine, showing a clear physiologic effect of FeLV-A infection.

The 61E strain of FeLV-A employed in our study only uses THTR1 as a receptor for cell entry, but it is clear that other subgroups of FeLV that evolve in FeLV-A-infected cats can use a wide range of related transporters as receptors (Fig. 1). These include the heme transporters FLVCR1 and FLVCR2 and the inorganic phosphate transporters Pit1 and Pit2. Even these divisions in receptor use between different FeLV subgroups are somewhat arbitrary, as one strain of FeLV has been identified that can utilize THTR1, FLVCR1, or FLVCR2 as a receptor for cell entry (31). Given this plasticity of receptor use, it would not be surprising if some strains of FeLV can utilize THTR2 as a receptor, leading to further disruption of thiamine uptake.

The expression patterns of feTHTR1 and feTHTR2 that we observed in feline tissues are generally consistent with those of THTR1 and THTR2 orthologs in humans and mice; THTR1 is

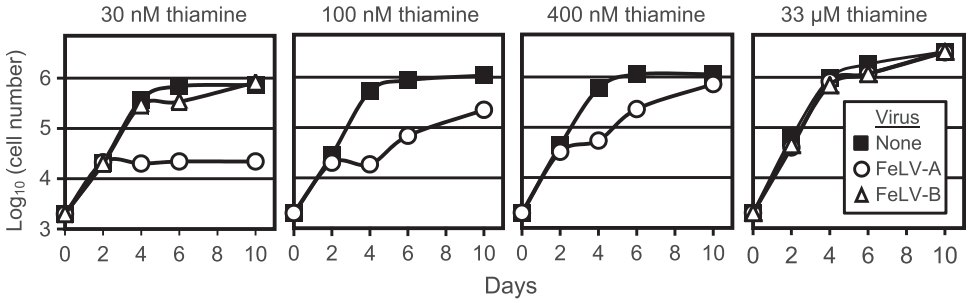

FIG 7 Growth of cells that depend on feTHTR1 for thiamine uptake is inhibited by FeLV-A infection. AH927 cells and their derivatives were seeded at 2×10^3 cells per well in multiple 6-well plates in thiamine-deficient medium supplemented with the indicated concentrations of thiamine. The medium was replaced every 2 days to maintain these thiamine concentrations. Cell numbers were measured by washing the cells two times with phosphate-buffered saline, suspending the cells by trypsin treatment, and counting cells that exclude trypan blue (live cells). Note that the cells become confluent and replication slows when the cell number reaches about 10^6 cells per well. Data are the means of two independent measurements made in the same experiment.

widely expressed, whereas THTR2 is more restricted in its expression. Ubiquitous expression of feTHTR1 has also been described in cats (40). We were able to detect feTHTR1 in every cell line and tissue that we tested. The broad tissue distribution of feTHTR1 is consistent with its role in mediating FeLV-A infection of multiple tissues and its broad feline host cell specificity. FeLV-A has been isolated from brain, thymus, lymph node, epithelial cells of the salivary gland, and intestine tissues of infected animals, all tissues in which we detected feTHTR1. Our data indicate that there are cells and tissues in cats that express only one thiamine transport protein, feTHTR1, and we expect that such cells will be more susceptible to the pathogenic effects of FeLV-A infection.

The growth of cells infected with FeLV-A at physiological concentrations of thiamine (median value, ≤30 nM; see Results for calculation) was decreased compared to control cells. Even at concentrations as high as 400 nM, well above what would be expected in a feline host, FeLV-A-infected cells still exhibited decreased growth rates compared to uninfected cells. No difference in growth rate between FeLV-A-infected cells and control cells was seen at very high levels of thiamine (33 μM), indicating that passive diffusion or low-affinity transport of the molecule is sufficient to rescue the defect in thiamine transport at high thiamine concentrations. This may explain why FeLV-A infection does not cause notable effects on cell viability under standard tissue culture conditions, where thiamine levels are high (e.g., 12 μM for DMEM).

The specific pathogenesis of FeLV-A has been difficult to define, as the virus often evolves new receptor specificities associated with new subgroups (5–7). This has made it difficult to tease out the specific contribution of FeLV-A to pathogenesis. However, FeLV infection among wild cats of the *Felidae* family has provided a window into the pathogenicity of FeLV-A. In a study of the prevalence and importance of known feline pathogens in critically endangered, free-ranging Iberian lynx (*Lynx pardinus*) (41), 6 of 11 lynx found to be infected with an FeLV-A subgroup virus (DNA and p27 antigen positive) died in a 6-month period. Death in the study population was strongly correlated with FeLV-A infection ($P < 0.001$), no endogenous FeLV sequences were detected in Iberian lynx that might give rise to more pathogenic FeLV recombinants, and no other viral pathogens, including FeLV-B and -C, were detected in the FeLV-infected animals. FeLV infection was associated with anemia, lymphopenia, and neutropenia. Sequencing of the FeLV surface glycoprotein genes in the FeLV-infected animals revealed nearly identical viruses that were closely related to the 61E strain of FeLV-A and most likely arose from virus transmitted from a domestic cat. Similarly, genetic characterization of a Florida panther FeLV outbreak in which five FeLV antigen-positive animals died (42) revealed the presence of virus related to the domestic cat FeLV-A strain 945, there was no evidence of endogenous FeLV sequences in the panthers, and pathogenic outcomes were related to FeLV infection. In this case, clinical symptoms included lymphadenopathy, anemia, septicemia, and weight loss. These results establish the pathogenic potential of FeLV-A independent of other FeLV strains that arise in domestic cats.

While we found that FeLV-A infection could disrupt thiamine uptake at physiological thiamine concentrations and block cell division, this effect could be reversed in the presence of high levels of extracellular thiamine. Thus, if any of the pathological effects of FeLV-A infection result from such disruption, it may be possible to reverse the pathology by raising the thiamine concentration in extracellular fluids. Indeed, the anemia, pancytopenia, and, to some extent, diabetes observed in humans with defective THTR1 function can be effectively treated, without adverse effects, by oral or parenteral administration of 20 to 100 mg thiamine per day (15, 43, 44). Likewise, these same diseases, which are observed in THTR1 knockout mice given a low thiamine diet, can be reversed by feeding the mice a standard mouse chow that contains a high level of thiamine (45). Thus, our results predict that the simple addition of high levels of thiamine to the diet may ameliorate disease in FeLV-infected domestic and wild cats. Furthermore, if immunosuppression seen in FeLV-infected cats is in part the result of disruption of thiamine uptake and resultant poor growth of immune system cells, immune control of FeLV will likely be improved by thiamine supplementation. In combination with vaccination, thiamine supplementation may be particularly useful for preservation of endangered wild cats exposed to FeLV.

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